

AD _____

Award Number: DAMD17-02-1-0683

TITLE: Breast Cancer Prevention by Inducing Apoptosis in DCIS Using Breast Ductal Lavage

PRINCIPAL INVESTIGATOR: Patrick P. Koty, Ph.D.

CONTRACTING ORGANIZATION: Wake Forest University School of Medicine
Winston-Salem, NC 27157

REPORT DATE: September 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 01-09-2006		2. REPORT TYPE Annual		3. DATES COVERED 1 Sep 2005 – 31 Aug 2006	
4. TITLE AND SUBTITLE Breast Cancer Prevention by Inducing Apoptosis in DCIS Using Breast Ductal Lavage				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-02-1-0683	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Patrick P. Koty, Ph.D.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Wake Forest University School of Medicine Winston-Salem, NC 27157				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES Original contains colored plates: ALL DTIC reproductions will be in black and white.					
14. ABSTRACT Current prevention focuses on oral administration of chemopreventive agents which decreases breast cancer incidence but increases the risk for secondary treatment-induced disease. In addition, these chemopreventive agents may not be effective in preventing those lesions that are estrogen receptor (ER) negative. We hypothesize that programmed cell death is dysregulated in premalignant breast cells which permits these cells to avoid cell death. We are currently investigating whether treating premalignant breast cells with a molecular genetic-based agent may be effective alone or in concert with tamoxifen treatment to induce cell death in both ER-positive and ER-negative cells. Our preliminary studies indicate a DCIS primary explant cell line overexpresses the anti-apoptotic proteins Bcl-2 and Bcl-xL compared to normal breast tissue. Our initial studies also show an increase in programmed cell death in the DCIS primary explant cell line after treatment with an antisense bcl-2/bcl-xL and/or tamoxifen. Ultimately, we envision delivering genetic-based preventive agents and/or a chemopreventive agent directly to the breast ductal lobe of these high risks eliminating any potential for secondary treatment-induced disease.					
15. SUBJECT TERMS Chemoprevention, apoptosis, breast ductal lavage, ductal carcinoma in situ, bcl-2					
16. SECURITY CLASSIFICATION OF:				18. NUMBER OF PAGES 9	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	9
Conclusions.....	9
References.....	9
Appendices.....	9

TITLE:

Breast Cancer Prevention by Inducing Apoptosis in DCIS Using Breast Ductal Lavage

INTRODUCTION:

Although great strides have been made in breast cancer screening and treatment, it remains the second highest cause of cancer-related deaths for women in the United States. Current prevention has focused on oral administration of chemopreventive agents which appears to decrease breast cancer incidence but increases the risk for secondary treatment-induced disease. In addition, these chemopreventive agents may not be effective in preventing those lesions that are estrogen receptor (ER) negative based on its primary function of suppressing cell proliferation by blocking the estrogen receptor. We hypothesize that programmed cell death (PCD) is dysregulated in premalignant and malignant breast cells which permits both ER-positive and ER-negative cells to avoid cell death. We intend to investigate whether treating premalignant breast cells with a molecular genetic-based agent (antisense bcl-2/bcl-xL oligonucleotide) may be effective alone or in concert with tamoxifen treatment to induce cell death in both ER-positive and ER-negative cells. Ultimately, we envision using the newly developed technique of breast ductal lavage to not only screen women for increased risk in developing breast cancer, which is currently being performed, but to also use this technique to delivery genetic-based preventive agents and/or a chemopreventive agent directly to the breast ductal lobe of these high risk individuals thus eliminating any potential for secondary treatment-induced diseases.

BODY:**Approval for the Use of Human Anatomical Substances**

Received approval from the DoD's Human Subject Protection Office for the use of human anatomical substances to obtain breast ductal lavage samples on **March 12, 2006**.

Received approval from the DoD's Human Subject Protection Office for the use of human anatomical substances to utilize five previously established primary explant DCIS cell lines and two previously established primary explant normal breast cell lines on **June 6, 2006**.

Approval for No Cost Extension

Received approval from the DoD on May 6, 2006 for a no cost extension for the period until August 31, 2007.

Statement of Work

- Task 1.* Determine expression pattern of the PCD regulatory genes bcl-2, bax, and bcl-xL in primary DCIS cultures (Months 1-6):
- a. Protein analysis of Bcl-2, Bax, and Bcl-xL using Western blotting and immunofluorescent staining.

I have recently received DoD approval (6-6-06) to work with the proposed five DCIS and two normal primary explant breast cell lines provided by Jean Latimer, PhD from the University of Pittsburgh Medical Center. At this time, Dr. Latimer has sent two DCIS cell lines (designated DCIS3 and DCIS4) and one normal breast cell line (designated BRL23). I have begun Western blot analysis to determine the endogenous protein levels for Bcl-2, Bcl-xL, and Bax. As can be seen in Figure 1, DCIS3 expresses both Bcl-2 and Bcl-xL but the normal breast cell line BRL23 does not express any detectable amounts of either protein.

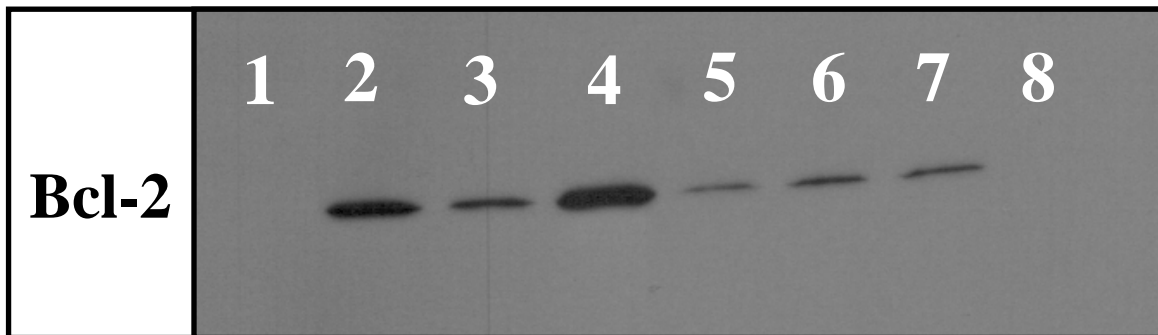
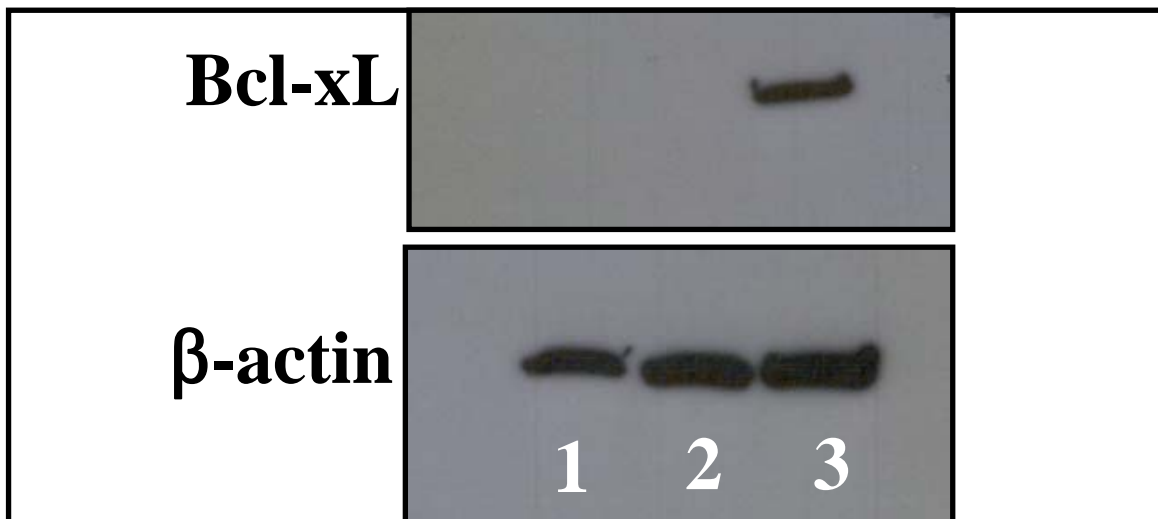
A**B**

Figure 1. Western blot analysis of endogenous levels of Bcl-2 and Bcl-xL. Equal amounts of total protein (50µg), as measured by spectroscopy using a standard protocol, was loaded into each lane of a 4-20% gradient polyacrylamide gel. Following protein transfer to a PVDF membrane, the blots were incubated in primary antibody (Bcl2 1:500, Dako; Bcl-xL 1:1000, Zymed; or β-actin 1:2000, Chemicon) overnight at 4° followed by incubation with a HRP secondary antibody (1:1000, Dako) for 2 hrs at room temperature. Chemiluminescence staining (Pierce) was performed followed by exposure to autoradiograph film. **A.** lanes 1 and 8: normal breast primary explant cell line BRL23; lanes 2-7: DCIS3 primary explant cell line **B.** lanes 1 and 2: normal breast primary explant cell line BRL23; lanes 3: DCIS3 primary explant cell line.

This preliminary evidence supports our hypothesis that programmed cell death is dysregulated in DCIS as compared to normal breast tissue. This will allow us to perform *Task 2*. Hopefully, we will receive the additional three DCIS cell lines and one normal cell line from Dr. Latimer to complete *Task 1* and continue with *Task 2*.

Task 2. Determine whether down-regulation by genetic manipulation of the anti-apoptotic genes bcl-2 and/or bcl-xL alone or in conjunction with physiological preventive doses of tamoxifen has the highest induction of PCD in primary DCIS cell cultures (Months 6-18):

- a. Treatment with antisense and control oligonucleotides and/or tamoxifen.
- b. Protein analysis of Bcl-2, Bax, and Bcl-xL using Western blotting and immunofluorescent staining.
- c. Quantify mRNA for bcl-2 or bcl-xL using a PCR-based assay.
- d. Determine effect of treatment on programmed cell death markers using assays for DNA fragmentation and caspase activation.

We have also begun to complete *Task 2* using the DCIS primary explant cell line that we have received. Our initial studies, based on the preliminary data obtained in *Task 1*, involves treating the DCIS cells with an antisense bcl-2/bcl-xl oligonucleotide and/or 4-hydroxytamoxifen (Sigma). We then determined the effect of this treatment on the programmed cell death pathway by performing a pan caspase activation assay (Immunochemistry Technologies) using a fluorescent microscopy protocol which we determined both caspase activation and the presence of apoptotic bodies (Figure 2).

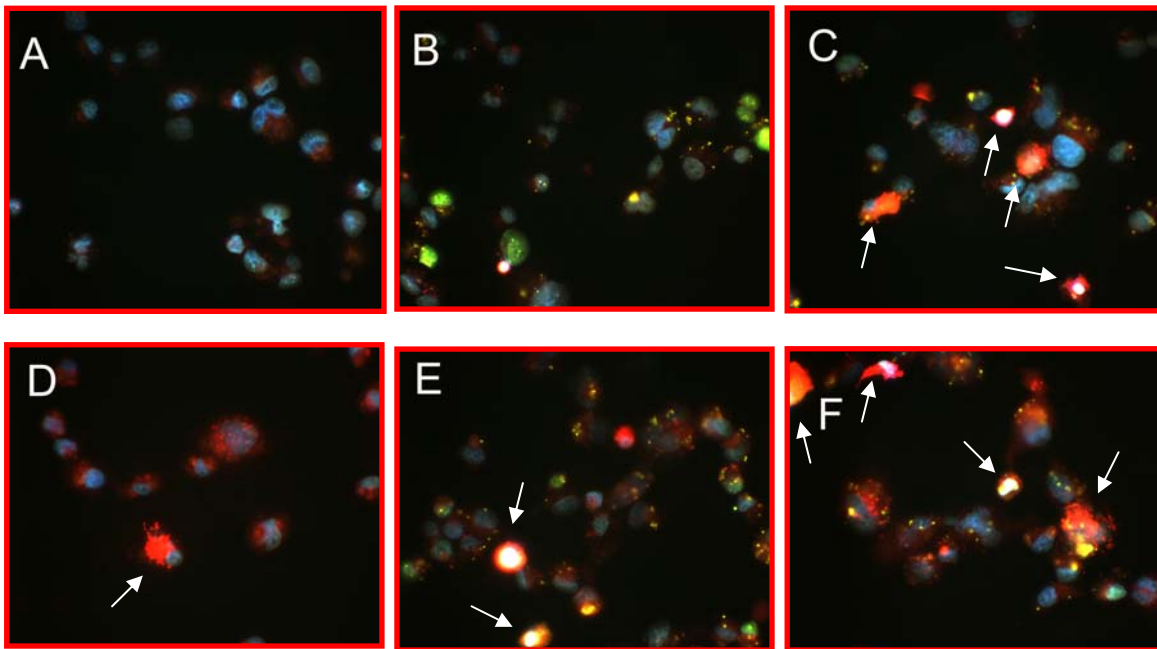


Figure 2. Induction of programmed cell death in the DCIS3 primary explant cell line after treatment with an antisense bcl-2/bcl-xl oligonucleotide and/or 4-hydroxytamoxifen. DCIS3 cells were treated with 0.5 μ g nonsense control or antisense bcl-2/bcl-xl oligonucleotides for 24 hrs then treated with 5 μ M 4-hydroxytamoxifen for an additional 24 hrs. Nuclei are stained with Hoechst 33342 (blue), oligonucleotides are

labeled with Fam (green), caspase activation identified by VAD cleavage (red) and indicated with arrows. **A.** Medium control **B.** nonsense oligonucleotide **C.** antisense bcl-2/bcl-xl oligonucleotide **D.** 4-hydroxytamoxifen **E.** nonsense oligonucleotide and 4-hydroxytamoxifen **F.** antisense bcl-2/bcl-xl oligonucleotide and 4-hydroxytamoxifen.

The results of this preliminary analysis are summarized in Table 1. This analysis indicates that all treatment conditions, except medium control, induce an increase in programmed cell death with the highest induction with treatment of antisense bcl-2/bcl-xl only. These preliminary studies need to be repeated and performed at different time points but do show promise as a potential preventative therapy by inducing programmed cell death in DCIS. We will also continue to complete *Task 2*.

Table 1.

Treatment Condition	Transfection Efficiency (%)	Caspase Activation and/or Apoptotic Bodies (%)
Medium control	NA	0
Nonsense oligonucleotide control	76	7.6
Antisense bcl-2/bclxl oligonucleotide	86	18.0
4-hydroxytamoxifen	NA	6.9
Nonsense oligo & 4-hydroxytamoxifen	87	11.9
Antisense oligo & 4-hydroxytamoxifen	78	12.0

NA – Not appropriate

Task 3. Determine expression pattern of the PCD regulatory genes bcl-2, bax, and bcl-xL in cells obtained by breast ductal lavage (Months 18-24):

- a. Protein analysis of Bcl-2, Bax, and Bcl-xL using immunofluorescent staining.

Breast ductal lavage samples were obtained from twenty-one informed patients. Unfortunately, the vast majority of these lavage samples had a very limited number of cells (at most hundreds) to evaluate and most of those cells were not ductal in origin. Most cells obtained have been squamous epithelium from the nipple, red blood cells, and macrophages and/or histiocytes (Figure 3). Currently, we have temporarily put this task on hold to focus our efforts on *Tasks 1* and *2*. However, I have been in consultation with the surgeons performing the ductal lavage procedure and the company (Cytoc) who manufactures the devices used in the lavage procedure. We are hopeful to rectify this problem shortly and to be able to successfully obtain sufficient cell numbers for *Task 3* and *4*.

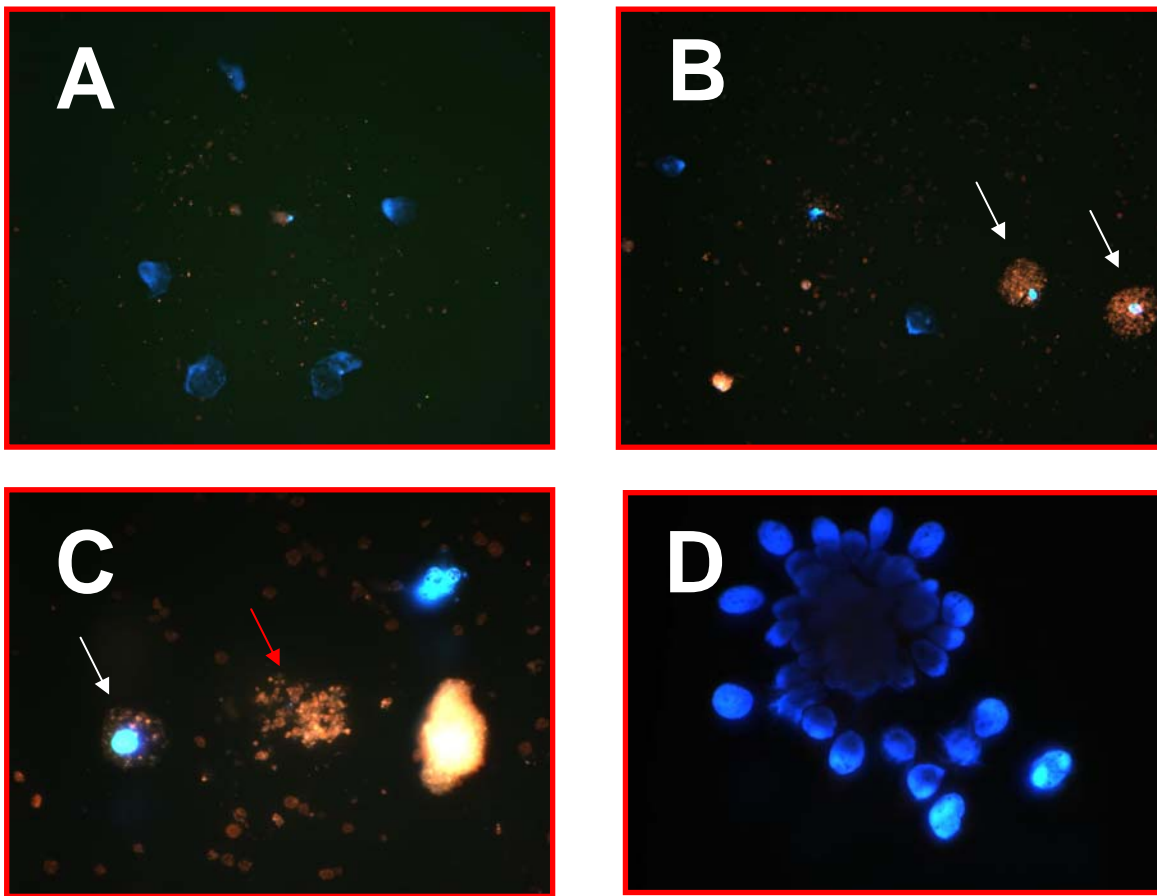


Figure 3. Fluorescence microscopic analysis of cells obtained from ductal lavage procedure. Nuclei were stained with DAPI. **A.** Squamous epithelium from nipple. **B.** Histiocytes (white arrow). **C.** Macrophage (white arrow) and red blood cells (red arrow). **D.** Breast duct epithelial cells.

Task 4. Determine whether down-regulation by genetic manipulation of the anti-apoptotic genes bcl-2 and/or bcl-xL alone or in conjunction with physiological preventive doses of tamoxifen has the highest induction of PCD in cells from breast ductal lavages (Months 24-36).

- a. Treatment with antisense and control oligonucleotides and/or tamoxifen.
- b. Protein analysis of Bcl-2, Bax, and Bcl-xL using Western blotting and immunofluorescent staining.
- c. Quantify mRNA for bcl-2 or bcl-xL using a PCR-based assay.
- d. Determine effect of treatment on programmed cell death markers using assays for DNA fragmentation and caspase activation.

KEY RESEARCH ACCOMPLISHMENTS:

- Shown the anti-apoptotic proteins Bcl-2 and Bcl-xL are overexpressed in DCIS cells as compared to normal breast tissue.
- Treatment of DCIS cells with antisense bcl-2/bcl-xL oligonucleotides and/or 4-hydroxytamoxifen induces programmed cell death.

REPORTABLE OUTCOMES: N/A**CONCLUSIONS:**

After receiving DoD approval to use the DCIS and normal breast primary explant cells, we have been able to begin *Tasks 1* and *2*. These initial studies support our hypothesis by showing that DCIS cells have a dysregulated programmed cell death pathway and are overexpressing Bcl-2 and Bcl-xL compared to normal breast tissue. We have also shown with this initial data that treatment of DCIS cells with antisense bcl-2/bcl-xl oligonucleotides and/or 4-hydroxytamoxifen induces programmed cell death. We anticipate the successful completion of *Tasks 1* and *2* once Dr. Latimer supplies the additional three DCIS and one normal breast tissue explant cell lines. We also expect to overcome the technical problem regarding obtaining sufficient cell number and cell types with the ductal lavage procedure to accomplish *Task 3* and *4*.

REFERENCES: N/A**APPENDICES: N/A**